IDENTITY AND PHYLOGENETIC PLACEMENT OF *SPIROGYRA* SPECIES (ZYGNEMATOPHYCEAE, CHAROPHYTA) FROM CALIFORNIA STREAMS AND ELSEWHERE[1]

Rosalina Stancheva[2]

Department of Biological Sciences, California State University San Marcos, San Marcos, California 92096, USA

John D. Hall

Department of Botany, Academy of Natural Sciences, Philadelphia, Pennsylvania 19103, USA

Richard M. McCourt

Department of Botany, Academy of Natural Sciences, Philadelphia, Pennsylvania 19103, USA
Department of Biodiversity, Earth, and Environmental Sciences, Drexel University, Philadelphia, Pennsylvania 19104, USA

and Robert G. Sheath

Department of Biological Sciences, California State University San Marcos, San Marcos, California 92096, USA

Diversity of the filamentous green algae in the genus *Spirogyra* (Zygnematophyceae) was investigated from more than 1,200 stream samples from California. We identified 12 species of *Spirogyra* not previously known for California (CA), including two species new to science, *Spirogyra californica* sp. nov. and *Spirogyra juliana* sp. nov. Environmental preferences of the Californian species are discussed in the light of their restricted distribution to stream habitats with contrasting nutrient levels. We also investigated the systematic relationships of *Spirogyra* species from several continents using the chloroplast-encoded genes ribulose-1,5-bisphosphate carboxylase/hydrogenase large subunit (*rbcL*) and the beta subunit of the ATP synthase (*atpB*). Californian species were positioned in most major clades of *Spirogyra*. The phylogeny of *Spirogyra* and its taxonomic implications are discussed, such as the benefits of combining structural and molecular data for more accurate and consistent species identification. Considerable infraspecific genetic variation of globally distributed *Spirogyra* species was observed across continental scales. This finding suggests that structurally similar species from distant regions may be genetically dissimilar and that *Spirogyra* may contain a large number of cryptic species. Correlating the morphological and genetic variation within the genus will be a major challenge for future researchers.

Key index words: California; generic and infrageneric concept; morphology; *rbcL* and *atpB* phylogenies; reproduction; *Sirogonium*; sp. nov; *Spirogyra*; streams; Zygnematophyceae

Abbreviations: *atpB*, ATP synthase beta subunit 3; CA, California; *rbcL*, RUBISCO large subunit; SEM, scanning electron microscopy

Species of *Spirogyra* Link (Zygnematophyceae) are important components of many freshwater habitats. *Spirogyra* often grows in long filaments that sometimes form conspicuous tufts or mats in slow-moving water. These filaments provide a substratum for other microscopic species and habitat for many aquatic organisms (Hoshaw 1968). Larger herbivores, including insect larvae, snails, fish and turtles, also consume filamentous green algae, which makes *Spirogyra* an important primary producer in many aquatic food webs (Hoshaw 1968), especially under moderately eutrophic to mesotrophic conditions (Simons 1994). *Spirogyra* colonizes habitats with a wide range of total nitrogen and total phosphorus, water conductivity, and alkalinity (e.g., Simons and Van Beem 1990, Hainz et al. 2009).

In the course of investigations of freshwater algae from streams in CA, several species of *Spirogyra* were encountered. Although the literature on *Spirogyra* is rich in records of occurrence worldwide, the diversity and distribution of *Spirogyra* and other freshwater benthic algae in CA are poorly documented. To date, only about 10 species of *Spirogyra* and the related genus *Sirogonium* Kütz. have been reported from CA or are likely to occur there (Collins 1909, Smith 1950, Transeau 1951, Leland et al.1986, Vis and Sheath 1996, J. D. Hall and K. G. Karol, unpublished data). This is a relatively small number of species considering the overall diversity of the genus (Kadłubowska 1984), the diverse habitats of CA, and the ubiquity and diversity of *Spirogyra* in other regions. For instance, 23 *Spirogyra* species were

described from running and standing waters in Arizona (Rickert and Hoshaw 1968, 1972), and 30 *Spirogyra* species were reported from pools and ditches in the Netherlands (Simons 1994).

More than 381 species of *Spirogyra* and 16 species of *Sirogonium* have been described (Kadłubowska 1984). Species are identified morphologically using a suite of vegetative and reproductive characteristics. Important vegetative characteristics include the diameter of the filament and the number of chloroplasts as well as the number of turns of the chloroplast per cell (Transeau 1951, Randhawa 1959, Hoshaw 1968, Kadłubowska 1984, Rundina 1998). All characteristics of the reproductive process are important for species determination: characteristics of the gametangia and conjugation tube as well as characteristics of the mature zygospore walls (Transeau 1951, Randhawa 1959, Hoshaw 1968, Kadłubowska 1984, Rundina 1998). Species of *Spirogyra* and *Sirogonium* cannot be determined morphologically unless reproductive characteristics are known. Their vegetative similarities can make identification difficult since non-reproductive filaments can occur throughout the year whereas reproductive filaments are rarely encountered (Transeau 1951).

Phylogenetically, *Spirogyra* (and the related genus *Sirogonium*) represents an early branching lineage within the conjugating green algae (Zygnematophyceae), although the exact placement of the genus within the Zygnematophyceae was not strongly supported statistically in previous studies (Gontcharov et al. 2004, Drummond et al. 2005, Hall et al. 2008). Regardless of its placement, *Spirogyra* was generally positioned on a relatively long branch (with respect to other lineages of conjugating green algae) and in the case of the nuclear ribosomal small subunit DNA on an extremely long branch (Gontcharov et al. 2004). *Spirogyra* may include the related genus *Sirogonium* (Gontcharov et al. 2004, Hall et al. 2008), which has sometimes been classified as a subgenus of *Spirogyra* (Czurda 1932). Some phylogenetic studies found *Sirogonium* to be sister to *Spirogyra* (if one included *Sirogonium*-like species such as *Spirogyra maxima*; Drummond et al. 2005), whereas other studies indicated that *Sirogonium* was embedded in a clade of *Spirogyra* species (Gontcharov et al. 2004, Kim et al. 2006, Hall et al. 2008). However, previous studies did not include the entire taxonomic or structural diversity of the genus *Spirogyra*.

Several formal and informal subgeneric classifications of *Spirogyra* have been proposed. These classifications were based on both reproductive and vegetative characters. For example, the classification in Kadłubowska (1984) treated *Sirogonium* and *Spirogyra* as distinct genera and divided *Spirogyra* into three sections: *Conjugata* (Vaucher) Hansg., *Colligata* Kadłub. and *Salmacis* (Bory) Hansg. Section *Conjugata* included most of the species of *Spirogyra*, section *Colligata* included those species with an external ring of wall material at the cell junctions, and section *Salmacis* included those taxa with replicate (folded) transverse walls between adjacent cells. Section *Conjugata* was subdivided into "groups" based on the number of chloroplasts and the presence or absence of ornamentation on the zygospores (Kadłubowska 1984). However, neither the subgenera nor the groups have been widely used in the taxonomic literature.

This article tests the phylogenetic relationships among *Spirogyra* species using a large sampling of *Spirogyra* in a molecular phylogenetic framework. We present detailed morphological characterization of two newly described *Spirogyra* species and discuss the taxonomic and ecological implications of our findings.

MATERIALS AND METHODS

Sample collection. This study summarized the biodiversity of *Spirogyra* from California, based on nearly 1,200 samples collected across the state from perennial and non-perennial streams in the spring, summer and fall of 2007–2010 as a part of projects sponsored by the California Water Board (map of all nine watershed regions sampled in this study can be found at: http://www.waterboards.ca.gov/water_issues/programs/peer_review/). One sample originated from a lake in an urban area in San Marcos. Samples were collected after Fetscher et al. (2009) and fixed in the field with 2.5% histological-grade glutaraldehyde (Sheath and Cole 1992). Water temperature, conductivity, and pH were recorded for each site using field meters (OAKTON Instruments, Vernon Hills, IL, USA). For dissolved inorganic nutrients, such as total dissolved nitrogen and total dissolved phosphorus, stream water samples were filtered using 0.45 μm pore-size glass fiber filters (MILLIPORE IRELAND Ltd., Cork, Ireland). Total dissolved nitrogen and total dissolved phosphorus were measured after USGS I-2650-03 (Patton and Kryskalla 2003) at University of Georgia, Odum School of Ecology Analytical Chemistry Lab. In addition to the fixed benthic algal samples, fresh qualitative samples were collected simultaneously at each stream site. Numerous stream sites were resampled in order to obtain reproductive *Spirogyra* filaments. Filamentous algae were collected by hand and kept at 4°C until processed in the laboratory. Samples containing reproductive filaments of *Spirogyra* were incubated in water from the habitat, filtered and diluted with distilled water for further intervals to complete sexual or asexual reproduction and to obtain mature spores. Samples were placed in the northern window of the laboratory at room temperature (held constant at 20°C). Reproductive filaments were checked every 3 d, until completely developed zygospores were observed and documented.

Some specimens were cultured for further microscopic investigation and molecular studies. Individual filaments were removed from the field sample and rinsed several times in sterile medium. Strains were cultured in Bold's Basal Medium (Nichols and Bold 1965) at 12°C, all on a 12 h light:12 h dark cycle, or in Guillard's Woods Hole Medium IX enriched with sodium bicarbonate and vitamins (Nichols 1973, Andersen 2005, Hall et al. 2008) in a constant temperature chamber at 15°C. Sexually mature fixed voucher specimens were deposited in the University Herbarium at University of California (UC), Berkeley, USA.

Microscopic observation and species identification. Specimen observation and photomicrography were performed using an

Olympus microscope BX41 with an attached Olympus Micro-Fire S99809 digital camera (Olympus Imaging America Inc., Center Valley, PA, USA). The size ranges given in the descriptions are based on a minimum of 20 measurements of fresh specimens belonging to each population and were taken by Rincon image analysis software (Imaging Planet, Goleta, CA, USA). Photographed specimens were either living or treated with 10% KOH for distinguishing spore wall layers.

The chemical composition of the sporangial wall was examined using cytochemical techniques for the localization of pectic substances (Ruthenium Red, Fisher Scientific, Pittsburg, PA, USA; Jensen 1962). Preparation of spores for SEM was performed following Hull et al. (1985). Spores were gold coated and observed by FEI Quanta 600 FEG SEM (FEI, North America NanoPort, Hillsboro, OR, USA) and by Hitachi S-2700 SEM (Hitachi High Technologies America, Inc., Pleasanton, CA, USA). The exospore was removed manually. Original material of *S. notabilis* Taft collected from the type locality in Texas by Taft, samples 76 and 77, stored in the Transeau Collection (Academy of Natural Sciences, Philadelphia) was consulted. The main taxonomic sources were Transeau (1951), Randhawa (1959), Gauthier-Lièvre (1965), Kadłubowska (1984), Rundina (1998), and Johnson (2011).

Phylogenetic estimation. Strains used in this study were requested from public culture collections or isolated from field collections made over the course of many years (Table S1 in the Supporting Information). DNA was extracted from cultured material using the Phytopure Plant DNA extraction kit (GE Healthcare, Pewaukee, WI, USA). Fragments of the chloroplast genes encoding *rbcL* and *atpB* were amplified by PCR. We used the GoTAq Green Master Mix (Promega Corporation, Madison, WI, USA) for PCR with a Mg concentration of 4.0 mM. The *rbcL* fragment was amplified using the primers of Hall et al. (2008). The *atpB* fragment was amplified by two rounds of PCR with the external set *atpB*-175FZYG 5′-TRTWACYTGTG ARGTACARCA-3′ and *atpB*-1404RZYG 5′-CYARRTARAACGCY TGTTCTGG-3′ and the internal primers *atpB*-700F 5′-TATGGTCAAATGAATGAA-CC-3′ and *atpB*-866R 5′-CCWACTGCAGAAGGCATAC-3′. The *atpB* gene was first amplified using 35 cycles of: 95°C for 20 s; 50°C for 20 s; 72°C for 45 s. The second round of PCR (using primers 175FZYG+866R and 700F+1404RZYG) followed the same cycling protocol except that the annealing temperature was lowered to 48°C. All fragments were sequenced at the University of Washington Genome Sequencing Center (Seattle, WA, USA) or Functional Biosciences (Madison, WI, USA). Sequences of *rbcL* from *Spirogyra* species were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank). Only unique sequences with at least some structural or geographic information were retained (Table S2 in the Supporting Information). Sequences were assembled and edited in Sequencher v. 4.10.1 (Gene Codes, Ann Arbor, MI, USA) and aligned using the translated amino acid sequences in MacClade (Maddison and Maddison 2000).

The *atpB* and *rbcL* fragments were aligned and analyzed separately. The topologies were visually checked for agreement and the *rbcL* data set was subsampled to match the taxa in the *atpB* data set. The combined data set was aligned and analyzed with a single partition and with the data set partitioned by gene. The *atpB* alignment was 1,207 nucleotides in length with 549 variable and 513 parsimony informative sites. The *rbcL* alignment was 1,335 nucleotides in length with 576 variable and 523 parsimony informative sites. The model was chosen using MrModeltest v. 2.3 (Nylander 2004). Alignments were analyzed under Likelihood using RAxML v. 7.2.8 (Stamatakis et al. 2008) through the CIPRES Science Gateway v. 3.1 (www.phylo.org/portal2) using the GTRGAMMA + I model for final tree estimation. Bootstrap analysis was performed using RAxML and allowing RAxML to determine the
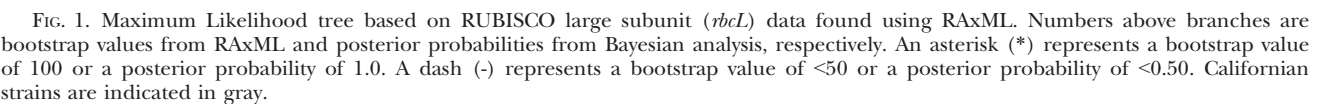
appropriate number of bootstrap pseudoreplicates (300 for the *rbcL* analysis and 200 for the two-gene analysis). Alignments were also analyzed using Bayesian Inference in MrBayes v. 3.2 (Ronquist and Huelsenbeck 2003). In Bayesian analyses, a model with six rate categories with parameters for the distribution of rate categories and invariant sites (GTR+I+G) was used. Two parallel runs with six chains were run for 10 million generations with the chains sampled every 1,000 generations. Tree topologies were summarized using the sumt command with the first quarter of the trees discarded as burnin. In the combined analysis, parsimony trees were estimated using a heuristic search with 100 random taxon additions in PAUP * v.4 beta 10 (Swofford 2003). Support was estimated using 500 bootstrap pseudoreplicates. The combined data set also was partitioned by gene and using the same models and parameters (Figure S1 in the Supporting Information).

RESULTS

Spirogyra of California. Fifteen species of *Spirogyra* were identified from studied streams, twelve of which were new records for CA, including two new *Spirogyra* species. *Spirogyra longata* (Vaucher) Kütz., *S. maxima* (Hassall) Wittr. and *S. varians* (Hassall) Kütz. were the only previously reported taxa encountered. Twenty-nine natural populations of *Spirogyra* were sequenced for both *rbcL* and *atpB*. Data on vegetative and reproductive characteristics of the strains and collection locality information are presented in Table S2. Unidentified strains from other regions were also included to provide context for understanding the distribution of genetic and species diversity.

Phylogeny of Spirogyra *and* Sirogonium. In the analyses of *rbcL*, several strongly supported lineages were discovered (Fig. 1). These are numbered I–VII for convenience and clarity (Fig. 1). Clade I contained all species with replicate transverse walls such as *S. australica* Czurda, *S. californica* Stancheva, J. D. Hall, McCourt et Sheath sp. nov., *S. croasdaleae* Blum, *S. grevilleana* (Hassall) Kütz., *S. tenuissima* (Hassall) Kütz., *S. weberi* Kütz. and strains JH0058 and JH0130 as well as some species and strains with plane transverse walls such as *S. borgeana* Transeau, *S. distenta* Transeau, *S. longata, S. lutetiana* P. Petit, *S. parvula* (Transeau) Czurda, *S. variformis* Transeau, JH0941, JH0980, and RSS020. Most species in this clade were relatively narrow (~10–50 μm in diameter) with one or two chloroplasts per cell. One notable exception was *S. californica*, which had 4–5 chloroplasts per cell and was 50–60 μm in diameter. Most strains had smooth mesospore membranes, but three species (*S. californica, S. australica,* and *S. croasdaleae*) had sculptured mesospore membranes.

Clade II contained species identified as *Sirogonium* and some species and strains of *Spirogyra* with multiple chloroplasts that made relatively few turns per cell. These taxa included *S. ellipsospora* Transeau, *S. maxima, Sirogonium melanosporum* (Randhawa) Transeau, *Sirogonium sticticum* (Sm.) Kütz., *Sirogonium tenuius* (Nordst.) Transeau as well as several

Fig. 1. Maximum Likelihood tree based on RUBISCO large subunit (*rbcL*) data found using RAxML. Numbers above branches are bootstrap values from RAxML and posterior probabilities from Bayesian analysis, respectively. An asterisk (*) represents a bootstrap value of 100 or a posterior probability of 1.0. A dash (-) represents a bootstrap value of <50 or a posterior probability of <0.50. Californian strains are indicated in gray.

unidentified strains. Filaments were generally thicker in diameter (32–150 μm) with 3–8 chloroplasts that generally made <1 turn per cell. Notable exceptions to these generalizations were unidentified strains JH0728 and JH0987 whose chloroplasts made 2–4 turns per cell, and strain JH0936 and RSS039, which had only 1 to 3 chloroplasts. Zygospores were ellipsoidal, or lenticular in *S. maxima.* Most strains had a smooth mesospore membrane, but *S. maxima* and *Sirogonium melanosporum* had sculptured mesospores.

Clade III contained two subclades of taxa. The first subclade consisted of *Spirogyra africana* (Fritsch) Czurda, *S. chungkingensis* C. C. Jao, *S. dubia* Kütz., *S. fluviatilis* Hilse, *S. mienningensis* L. Ching Li, *S. notabilis* Taft, *S. occidentalis* (Transeau) Czurda, *S. quadrilaminata* C. C. Jao, as well as several unidentified strains. These species had filament widths 30–57 μm, two to six or more chloroplasts (JH0278), and complex two-, or multi-layered sculptured mesospores, except for the smooth mesospore of *S. dubia.* The second subclade included *S. pratensis* Transeau UTEX1746 and two unidentified strains with filament width 15–20 μm and one or two chloroplasts. Chloroplasts generally made several turns per cell. Clade III was often allied with Clade IV, which contained *S. liana* Transeau, *S. pratensis* UTEX928 and strain JH0429. These strains were of small diameter (11–28 μm), had one chloroplast that made several turns per cell, and had a smooth mesospore membrane.

Clade V contained *S. communis* (Hassall) Kütz., *S. decimina* (O. F. Müll.) Dumort., *S. gracilis* Kütz., *S. teodoresci* Transeau, *S. varians* (Hassall) Kütz. and *S. juliana* Stancheva, J. D. Hall, McCourt et Sheath sp. nov., as well as several unidentified strains. These strains were 25–64 μm in diameter with one or two chloroplasts (two to four in *S. decimina*) that made several turns per cell; the mesospore was smooth or sculptured as in *S. juliana,* and some populations of *S. teodoresci* and *S. varians.* Two unidentified strains, JH0977 and ARL82-189, had almost identical *rbcL* sequences and formed an early branch within Clade V. *Spirogyra communis* UTEX 2463, five strains of *S. teodoresci* from CA and one identified strain, ARL 87-3, formed a separate clade. *Spirogyra varians* strains from CA, India (*S. varians* UTEX479) and South Korea (*S. varians* KCH 206-1 and KCH 96-4) were grouped together with *S. decimina, S. gracilis* and *S. juliana.*

Clade VI contained only two taxa, *S. condensata* (Vaucher) Dumort. and *S. juergensii* Kütz. Filaments of these species were of moderate diameter (24–60 μm) with one chloroplast that made one to four turns per cell and smooth mesospore. Both strains were isolated from Kanke, India.

Clade VII formed an early branch within the *Spirogyra* phylogeny. It contained *S. majuscula* Kütz., *S. submaxima* Transeau, and several unidentified strains (Fig. 1). These strains had a large cell diameter (80–108 μm), many chloroplasts (4–10 per cell) that typically made few turns per cell (0.5–1.5 turns), and often exhibited thick sheaths around the filaments. Zygospores were lenticular with smooth mesospores having a finely striated inner layer.

The deepest nodes of the *Spirogyra* clade in the *rbcL* analyses were only weakly supported (Fig. 1). The chloroplast-encoded gene *atpB* was amplified from a subset of 66 *Spirogyra* species representing most of the phyletic diversity within the genus. These two gene fragments were combined into a single data set. In the combined data set, the position of the taxa represented in the major clades did not change; within clades some branch rearrangements occurred, usually for branches that were supported at moderate statistical levels in Figure 1. Support for the deeper nodes for these major clades I–VII improved, although support for the placement of clades III–VI, relative to one another, was still quite low (Fig. 2). The topology of the nine most parsimonious trees and the best tree determined in RAxML were similar to one another with respect to the branching order of the major clades but differed from the topology observed using *rbcL* alone. The relative positions of clade V and clade III+IV were switched (Fig. 2). However, the deepest nodes in Figure 2 were not strongly supported statistically. The Bayesian consensus tree from the two-gene analysis differed considerably from the *rbcL* tree in that clades III, IV, V, and VI formed a monophyletic group sister to clade I (data not shown).

In both the *rbcL* and two-gene analyses the *Spirogyra*+*Sirogonium* clade was found to be sister to all other Zygnematophyceae (Figs. 1 and 2) although this relationship was not strongly supported in the two-gene analysis (Fig. 2). This relationship was also found in the Bayesian analysis of the *rbcL* data set (Fig. 1). In the Bayesian analysis of the two-gene data set, *Spirogyra*+*Sirogonium* was positioned sister to the Desmidiales clade with a posterior probability of 0.93 (data not shown).

Bayesian and RAxML analyses of the partitioned data set resulted in topologies very similar to the topology of the two-gene data set for the respective methods. Although partitioning the data set affected the statistical support of several clades, the differences were relatively small (Fig. S1).

Here, we present brief morphological descriptions, illustrations, and distributional data for all fifteen *Spirogyra* species identified from Californian streams. Species descriptions are based on a combination of vegetative and reproductive features, in particular the zygospore shape and wall structure and ornamentation, as well as conjugation tubes and gametangium characters. The listing of *Spirogyra* species follows the phylogenetic grouping seen in the *rbcL* analysis.

Clade I: **Spirogyra borgeana** Transeau (Fig. 3, A, B and M; Transeau 1951, p. 155, plates XXII, fig. 8, and
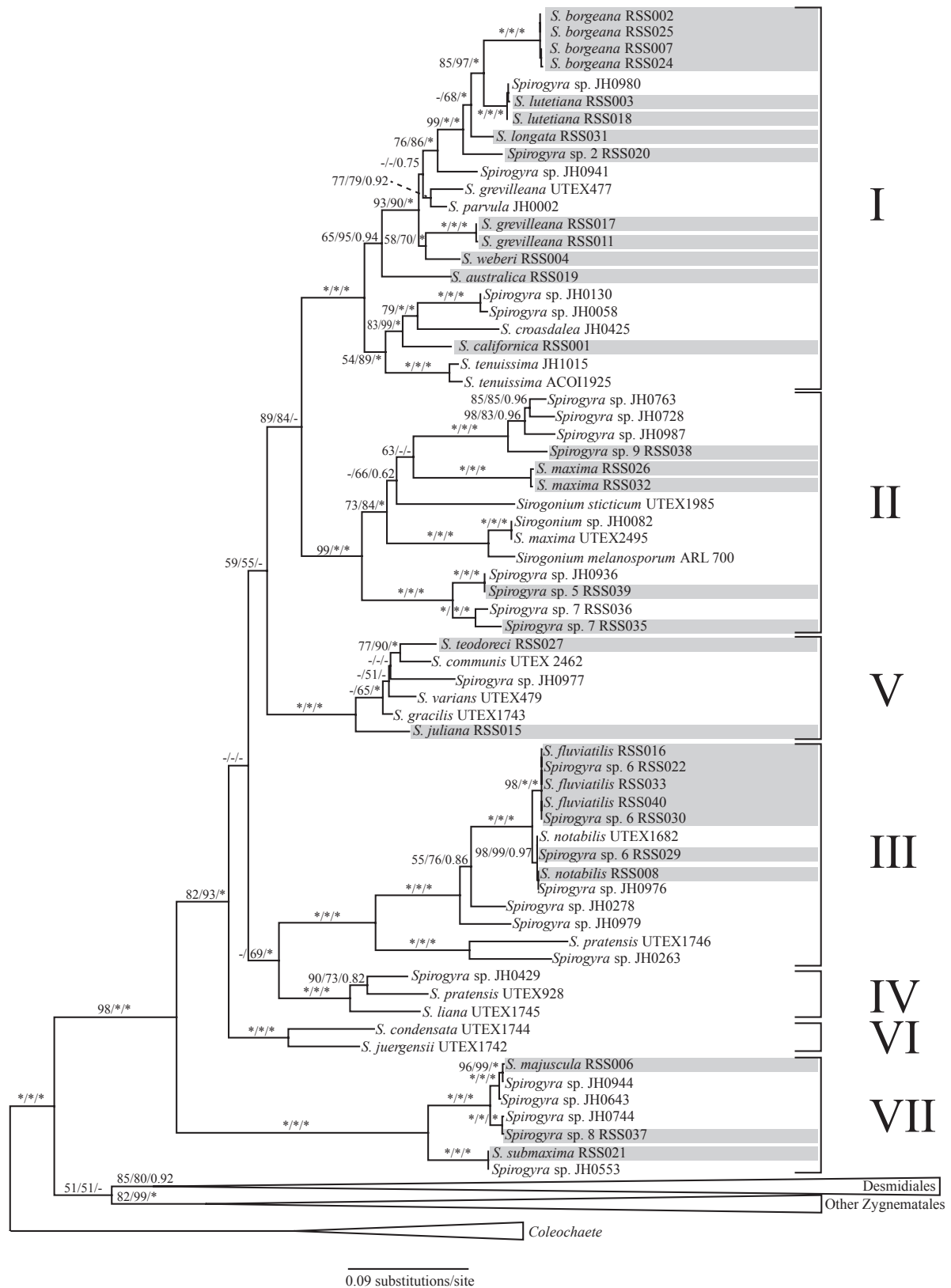
Fig. 2. Maximum Likelihood tree based on a combined analysis of ATP synthase beta subunit 3 (*atpB*) and RUBISCO large subunit (*rbcL*) found using RAxML. Numbers above branches are bootstrap values from Parsimony, RAxML and posterior probabilities from Bayesian analysis, respectively. An asterisk (*) represents a bootstrap value of 100 or a posterior probability of 1.0. A dash (-) represents a bootstrap value of <50 or a posterior probability of <0.50. Californian strains are indicated in gray.

XXIII, fig. 16, Gauthier-Lièvre 1965, p. 104, plate XXX, a, Kadłubowska 1984, p. 293, fig. 451, Rundina 1998, p. 189, fig. 78, 5–7, Johnson 2011, p. 589, plate 149 K).

Vegetative cells 26–33 μm wide, 58–331 μm long; transverse walls plane; chloroplast one or two per cell (Fig. 3A). Conjugation scalariform, tubes formed by both gametangia; fertile gametangia inflated on the outer side (Fig. 3B). Zygospores ellipsoid, 29–36 × 39–64 μm or sometimes spherical with diameter 33–40 μm, mesospore yellow-brown, smooth (Fig. 3M).

Notes: Spherical zygospores in this species had not been previously reported.

Distribution: Widely reported from Europe, Asia and United States (Transeau 1951, Jao 1988, Rundina 1998, Kargupta and Jha 2004). In studied area identified from thirteen localities in central and southern CA (watershed regions 4, 5 and 9).

Spirogyra lutetiana P. Petit (Fig. 3, C, D and N) (Transeau 1951, p. 160, plate XXIII, figs. 11–13, Gauthier-Lièvre 1965, p. 131, plate XXXXI, c, Kadłubowska 1984, p. 273, fig. 416, Rundina 1998, p. 182, figs. 75, 3, Johnson 2011, p. 597, plate 146 K).

Synonym: *Spirogyra decimina* f. *decimina* (O. F. Müll.) Kütz.

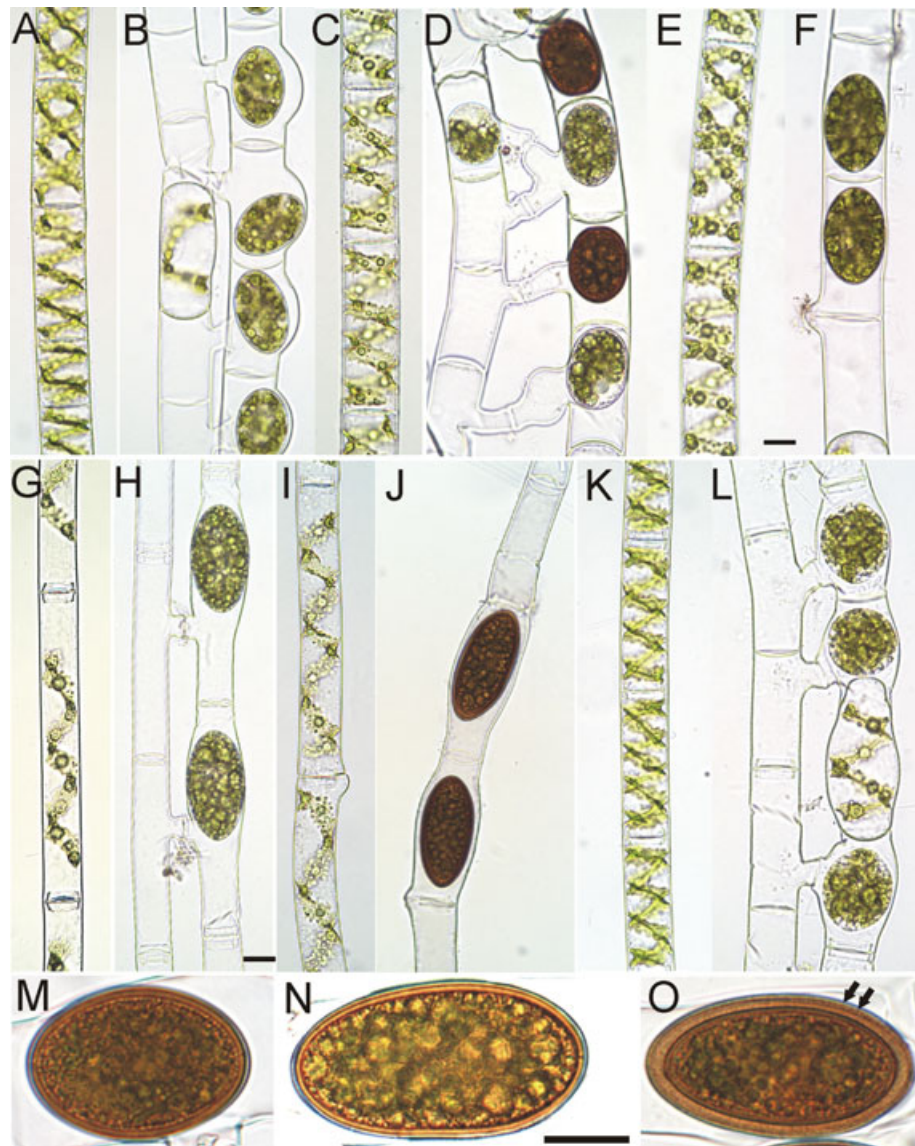Vegetative cells 26–40.5 μm wide, 63–309 μm long; transverse walls plane; chloroplast one or two

Fig. 3. Light microscopic images of *Spirogyra* species from CA included in Clade I of phylogenetic analysis: (A and B) *S. borgeana*; (C and D) *S. lutetiana*; (E and F) *S. longata*; (G and H) *S. weberi*; (I and J) *S. grevilleana*; (K and L) *S. australica*; (M) *S. borgeana* zygospore with smooth mesospore; (N) *S. lutetiana* zygospore with smooth mesospore; (O) *S. australica* zygospore with punctate mesospore (arrow). Scale bar, 20 μm.

per cell (Fig. 3C). Conjugation scalariform, rarely lateral, tubes long, somewhat inflated, formed by both gametangia; fertile gametangia cylindrical or slightly inflated on the outer side. Conjugation often happened between three cells and parthenospores are produced (Fig. 3D). Zygospores polymorphic, globose, ovoid, ellipsoid, cylindrical, often irregular with central inflation, 30–45 × 43–90 μm; mesospore yellow-brown, smooth (Fig. 3N).

Distribution: Widely reported from Europe, Asia, and known from United States (Transeau 1951, Jao 1988). Rare species in studied area, known from three localities in southern CA (watershed regions 9 and 4).

Spirogyra longata (Vaucher) Kütz. (Fig. 3, E and F) (Transeau 1951, p. 158, plate XXIII, fig. 5, Gauthier-Lièvre 1965, p. 130, plate XXXXII, a and b, Kadłubowska 1984, p. 267, fig. 409, Rundina 1998, p. 186, fig. 77, 3–4, Johnson 2011, p. 593, plate 146 D).

Synonym: *Spirogyra decimina* f. *longata* (Vaucher) V. I. Poljansky.

Vegetative cells 30–40 μm wide, 50–320 μm long; transverse walls plane; chloroplast one or two per cell (Fig. 3E). Conjugation predominantly lateral, rarely scalariform in the same filament with lateral conjugation, tubes formed by both gametangia; fertile gametangia cylindrical (Fig. 3F). Zygospores ellipsoid to ovoid 33–38 × 54–72 μm; mesospore yellow-brown, smooth.

Distribution: Widely distributed in the United States (Transeau 1951) and Europe with wide range of habitat tolerance (Johnson 2011); also reported from China (Jao 1988) and India (Kargupta and Jha 2004). Rare species in studied area, known from two urban stream localities in southern CA (watershed region 9).

Spirogyra grevilleana (Hassall) Kütz. (Fig. 3, I and J; Transeau 1951, p. 205, plates XXXV, figs. 19–20, Gauthier-Lièvre 1965, p. 172, plate LXV, d, Kadłubowska 1984, p. 453, fig. 703, Rundina 1998, p. 284, fig. 124, 4–6, Johnson 2011, p. 595, plate 150 B).

Synonym: *Spirogyra weberi* f. *grevilleana* (Hassall) V. I. Poljansky.

Vegetative cells 24–26 μm wide, 86–400 μm long; transverse walls replicate; chloroplast one per cell (Fig. 3I). Conjugation predominantly lateral; fertile gametangia slightly enlarged. Zygospores ovoid to cylindrical-ovoid 31–43 × 65–88 μm; mesospore yellow-brown, smooth (Fig. 3J).

Notes: We observed only lateral conjugation in studied populations, although both lateral and scalariform conjugation had been reported for this species.

Distribution: Widespread in Europe, Asia, and United States (Transeau 1951, Johnson 2011). Rare species in studied area, known from four localities in southern CA (watershed region 9).

Spirogyra weberi Kütz. (Fig. 3, G and H) (Transeau 1951, p. 205, plates XXXV, fig. 14, Gauthier-Lièvre 1965, p. 183, plate LXVIII, d–h, Kadłubowska 1984,

p. 446, fig. 191, Rundina 1998, p. 281, figs. 123, 124, Johnson 2011, p. 601, plate 146 E).

Vegetative cells 20–28 μm wide, 90–373 μm long; transverse walls replicate; chloroplast one per cell (Fig. 3G). Conjugation scalariform, tubes formed by both gametangia; fertile gametangia slightly enlarged (Fig. 3H). Zygospores ovoid to cylindrical-ovoid 28–35 × 65–79 μm; mesospore yellow-brown, smooth.

Notes: SEM observations showed a two-layered mesospore with outer layer thin and hyaline, and the inner layer thicker, smooth, pigmented (Simons et al. 1982).

Distribution: Widely reported from Europe, Asia and United States (Transeau 1951, Jao 1988, Johnson 2011). In the studied area, recorded in ten localities across CA (watershed regions 1, 4, 5, 6 and 9).

Spirogyra australica Czurda (Fig. 3, K, L and O) (Czurda 1932, p. 157, Reith 1988, p. 461, figs. IV, V, Rundina 1998, p. 299).

Vegetative cells 27–30 μm wide, 60–133 μm long; transverse walls replicate; chloroplast one or two per cell (Fig. 3K). Conjugation scalariform, tubes formed by both gametangia with slight predominance by the male gametangia; fertile gametangia enlarged to slightly inflated on both sides (Fig. 3L). Zygospores ellipsoid to ovoid 32–41 × 55–70 μm; mesospore 3–6 μm thick, brown, punctate (Fig. 3O).

Notes: Californian specimens corresponded well to the description of this species.

Distribution: This species was known from Germany, Australia (Reith 1988, Rundina 1998) and Spain (Alvárez Cobelas 1984). Czurda (1932) reported *S. australica* from Australia, but this information was vague. Rundina (1998) repeated the Australian distribution of this species, with the clarification that *S. australica* was poorly known and an original illustration was lacking. This species was rare in the studied area, known from a single mountain stream in southern CA. Our specimens of *S. australica* were very similar with Reith's (1988) descriptions and illustrations.

Spirogyra californica Stancheva, J. D. Hall, McCourt et Sheath **sp. nov.** (Fig. 4).

Vegetative cells 48–60 μm wide, 120–650 μm long, transverse walls replicate. Chloroplasts four (rarely three or five per cell), making 0.5–2 turns (Fig. 4A). Conjugation scalariform; short tubes formed by male gametangium connected to inflated middle portion of fertile receptive gametangium; fertile gametangia enlarged up to 85 μm, more on the conjugative side, outer margin slightly concave in the middle portion (Fig. 4B). Zygospores ellipsoid 55–69 μm wide, 95–107 μm long, almost filling the gametangia and placed along the longitudinal axis (Fig. 4B). Exospore smooth and colorless (Fig. 4, C and D). Mesospore two-layered, outer layer yellow-brown, translucent,
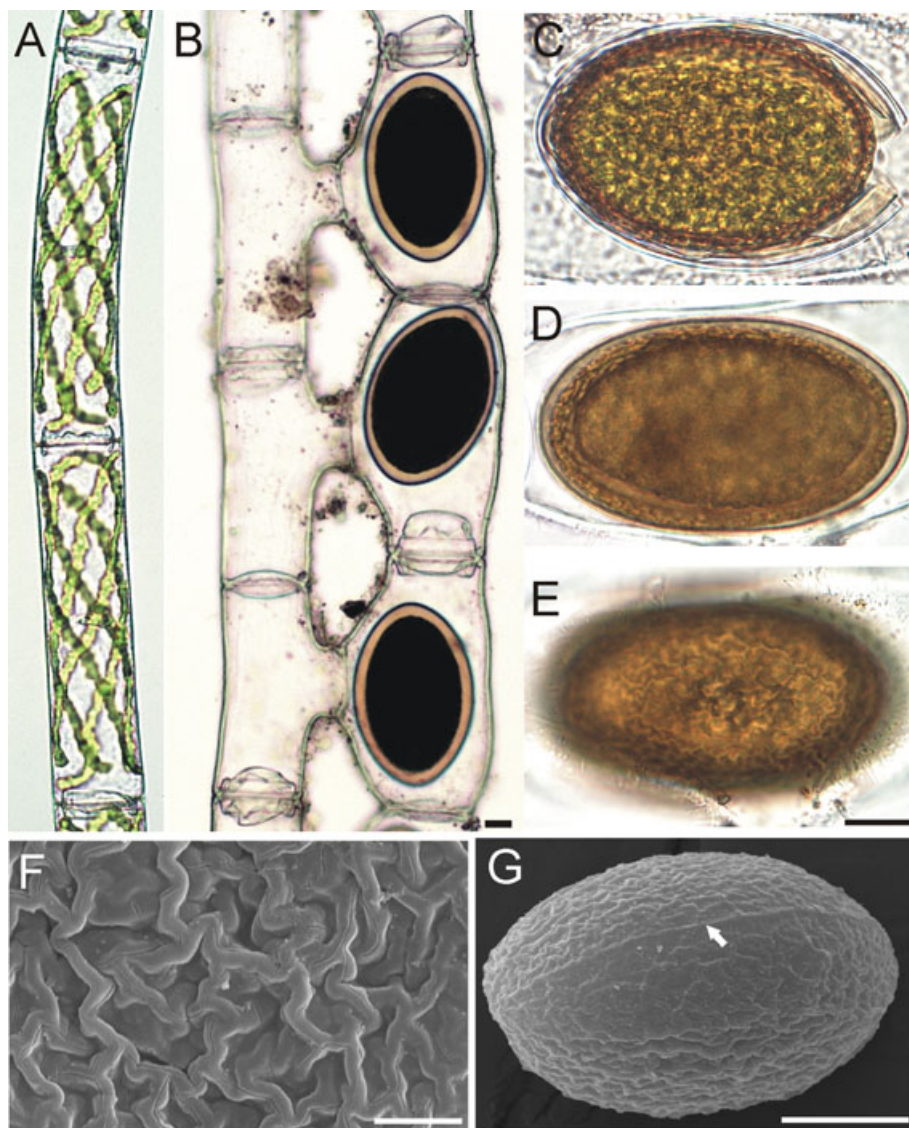
Fɪɢ. 4. Light microscopic and scanning electron microscope (SEM) images of *Spirogyra californica* included in Clade I of phylogenetic analysis: (A) filament with replicate transverse walls; (B) conjugation; (C) zygospore with colorless exospores, and yellow-brown, thin, and wrinkled outer mesospore layer; (D) zygospore focused on yellowish pectic layer accumulated between two mesospore layers; (E) same zygospore as above focused on surface of inner brown, reticulate mesospore layer with ridges; (F) SEM images of inner reticulate layer of mature zygospore with delicate fissures on top of ridges and small granules scattered over mesospore surface; (G) SEM images of inner reticulate layer with suture (arrow). Scale bar, (F) 5 μm; (A–G) 20 μm.

thin, wrinkled (Fig. 4C); inner layer brown, thick, reticulate with coarse ridges (Fig. 4, D and E); suture prominent (Fig. 4G). SEM images of the inner reticulate layer of mature zygospore showed delicate fissures on the top of the ridges and small granules scattered over the mesospore surface (Fig. 4F). The Ruthenium Red method revealed that at the beginning of zygospore development a pectic layer up to 7 μm in thickness was accumulated between the two mesospore layers (Fig. 4D). The amount of this pectic material decreased with zygospore aging. Parthenospores very rare, globose, 45–50 μm in diameter, mesospore same as in the zygospores.

Holotype: Specimen UC1999501 deposited in University Herbarium at University of California, Berkeley (UC).

Type locality: Medea Creek (34°11′ N, 118°75′ W), Malibu Creek watershed in the Malibu Creek State Park, Los Angeles County, CA, USA. July 02, 2007.

Type strain: RSS001, from culture.

Etymology: The epithet refers to the USA State of California, where *S. californica* was first observed.

Distribution and ecology. This species was found only in Malibu Creek watershed where it was recollected in the type locality in July 2008 and 2009, and in Malibu Creek downstream from the Malibu Lake, in which Medea Creek flows on June 26, 2012. Water

chemistry measurements in the type locality in 2007 and 2008 were pH 7.7–7.9; temperature 18.8°C–20.2°C; conductivity 2790–3143 $\mu S \cdot cm^{-1}$; total dissolved phosphorus 0.052–0.098 $mg \cdot L^{-1}$; total dissolved nitrogen 0.357–0.371 $mg \cdot L^{-1}$, sulfate 1016–1017 $mg \cdot L^{-1}$. *S. californica* was associated with *Cladophora glomerata* (L.) Kütz. and *Ulva flexuosa* Wulfen.

Related species. *S. californica* possesses a very rare combination of filament features: replicate cell walls, three to five chloroplasts per cell and conjugation tube formation by male gametangia. These characters were observed in *S. ionia* Wade, *S. rectangularis* Transeau and *S. insignis* (Hassall) Kütz., but these taxa have narrower filaments and different zygospore shape and sculpture. Overall, *S. crassivallicularis* C. C. Jao most closely resembled *S. californica* in respect to filament width, cells with replicate cell walls and usually four chloroplasts, as well as similar sculpture of two-layered mesospore, except for the lack of pectic material. However, *S. crassivallicularis* differs by the shape of zygospores and receptive gametangia, and by prominent conjugation tubes formed by both gametangia at their distal portion. Another similar species is *S. reticulata* Nordst., but the most striking differences were narrower filaments with one to four chloroplasts, lateral conjugation (Transeau 1951, Johnson 2011) and ultrastructure of the inner reticulate mesospore layer (Rundina 1998). For details see Table S3 in the Supporting Information.

Clade II: **Spirogyra maxima** (Hassall) Wittr. (Fig. 5, I–L) (Transeau 1951, p. 194, plate XXXII, figs. 10–11, Gauthier-Lièvre 1965, p. 134, plate XXXXIV, a and b, Kadłubowska 1984, p. 432, fig. 174, Rundina 1998, p. 270, figs. 117, 118, Johnson 2011, p. 597, plate 149 H).

Synonym: *Degagnya maxima* (Hassall) A. Conard.

Vegetative cells 120–150 µm wide, 90–280 µm long; transverse walls plane; chloroplasts 5 to 8 per cell (Fig. 5I). Conjugation scalariform, tubes formed by both gametangia; fertile gametangia short cylindrical (Fig. 5J). Zygospores lenticular, 113–150 × 113–150 × 72–95 µm; mesospore brown, multilayered, reticulate (Fig. 5K). Suture prominent, visible on the side-view (Fig. 5L).

Notes: SEM observations showed a three-layered mesospore with outer layer thin and hyaline, and two inner pigmented layers (Simons et al. 1982). The inner sublayer showed labyrinthine reticulated structure under SEM (Simons et al. 1982).

Distribution: Widely distributed in Europe, America, Asia, Africa, Australia (Transeau 1951, Johnson 2011). In the studied area, recorded in five localities in northern and southern CA (watershed regions 2 and 9).

Clade III: **Spirogyra notabilis** Taft (Fig. 6, A, B, E, F; Taft 1944, p. 238, Transeau 1951 p. 185, Rickert and Hoshaw 1968, p. 65, figs. 20–21).

Vegetative cells 33–35 µm wide, 100–309 µm long, transverse walls plane; chloroplasts 2–4 per cell (Fig. 6A); multicellular branched rhizoidal outgrowths present. Conjugation scalariform, tubes formed by both gametangia; cell wall thickened at conjugation. Fertile receptive gametangia enlarged near the zygospore, nearly cylindrical and slightly bent on the outer side (Fig. 6B). Zygospores ovoid to cylindrical-ovoid, 37–44 × 63–75 µm; occupied half to two-thirds of gametangia. Exospore smooth, colorless. Mesospore yellow-brown, two-layered, outer layer scrobiculate (Fig. 6E), inner layer thick, reticulate and finely verrucose with distinct, straight, diagonal suture (Fig. 6F).

Lectotype of *S. notabilis* Taft (here designated): Slide held at the Academy of Natural Sciences of Philadelphia (PH) from Taft sample 77 (PH01108521).

Notes: Taft (1944) described *S. notabilis* without providing illustrations of the type species and without designating a type specimen. We examined original material of *S. notabilis* including the above-designated lectotype, from the type locality and observed greater morphological variability. Therefore, we made two corrections in the original descriptions: (i) conjugation tubes formed by both gametangia, sometimes predominantly by the male gametangia; (ii) zygospore size range is extended to 30–57 × 46–105 µm. The Californian population of *S. notabilis* looks very similar to the type species, but only a few zygospores were recorded (Fig. 6, B, E, F), and therefore the zygospore size range was narrower.

Distribution: This species was described from Texas (Taft 1944), and further recorded in Arizona (Rickert and Hoshaw 1968) and India (Kargupta and Jha 2004, Anonymous 2012). In the studied area, we found this species reproducing only in a single locality in southern CA (watershed region 9).

Phylogenetic and taxonomic consideration: Molecular analysis showed that *S. notabilis* from CA is genetically identical to several species from Arizona: *S. notabilis*, *S. chungkingensis*, *S. mienningensis*, *S. occidentalis*, and *S. quadrilaminata* identified by Rickert and Hoshaw 1968. These species have in common a two-layered mesospore with reticulate-scrobiculate appearance, but otherwise their vegetative and reproductive morphology is highly variable in the following range: filament width 30–55 µm, chloroplasts 2 to 5 per cell, fertile gametangia cylindrical to enlarged, zygospores ovoid, ovoid-cylindrical to ellipsoid, 27–60 µm wide, 35–150 µm long. Based on reexamination of published and unpublished micrographs taken by Rickert and Hoshaw (1968), it seems possible that the material collected and described as different species in Arizona (i.e., *S. notabilis*, *S. chungkingensis*, *S. mienningensis*, *S. occidentalis*, and *S. quadrilaminata*) may all represent different developmental stages of one or two species (see Discussion).
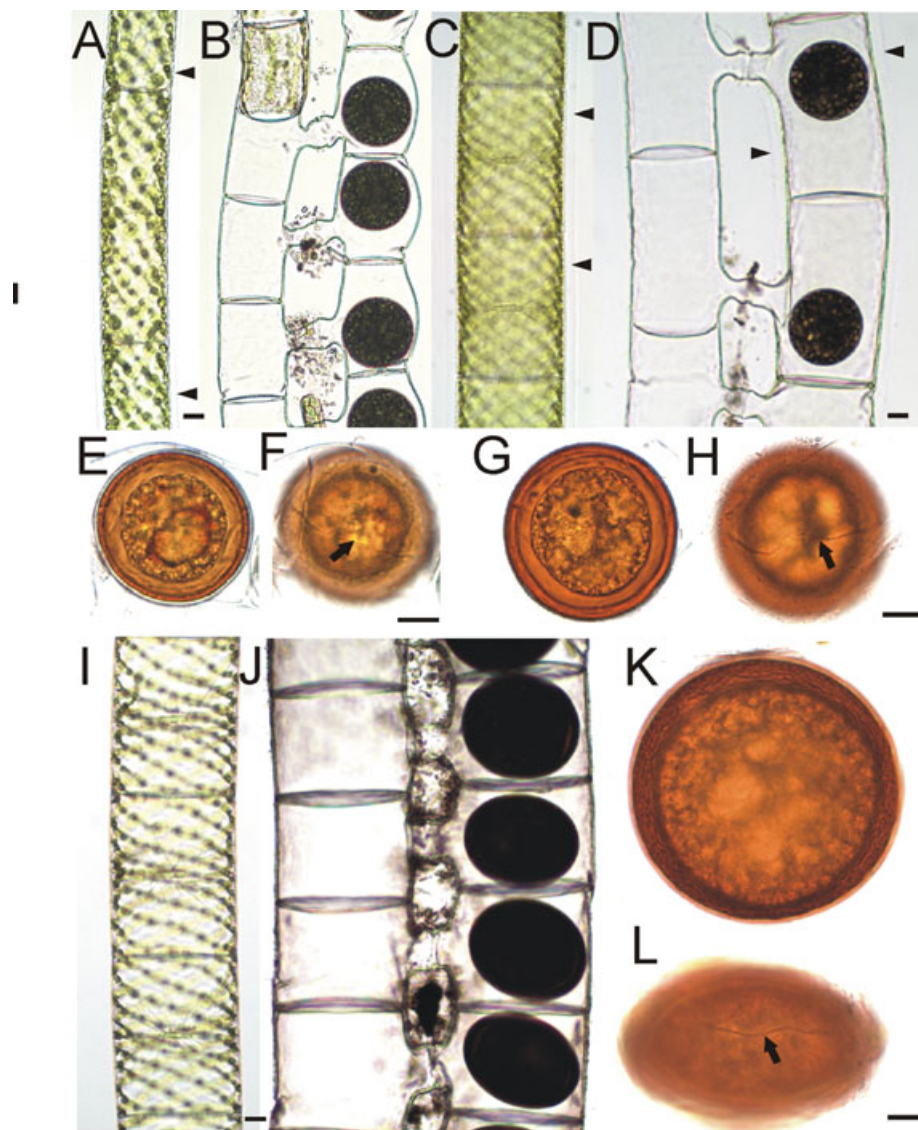
FIG. 5. Light microscopic images of *Spirogyra* species from CA included in Clades II and VII of the phylogenetic analysis: (A and B) *S. majuscula*; (C and D) *S. submaxima*; (A, C and D) Arrowheads show pectic layer that covers the filaments; (E) *S. majuscula* zygospore in front view with smooth brown mesospore with finely striated inner layer; (F) same zygospore as above focused on mesospore surface to show the suture (arrow); (G) *S. submaxima* zygospore in front view with smooth brown mesospore with finely striated inner layer; (H) same zygospore as above focused on mesospore surface to show the suture (arrow); (I–L) *S. maxima*; (I) filament; (J) conjugation; (K) zygospore in front view with brown multilayered reticulate mesospore, note lack of suture; (L) zygospore in side view focused on suture (arrow). Scale bar, 20 μm.

Spirogyra fluviatilis Hilse (Fig. 6, C, D, G) (Transeau 1951, p. 184, plate XXX, fig. 5, Gauthier-Lièvre 1965, p. 119, plate XXXVI, a, Kadłubowska 1984, p. 406, fig. 134, Rundina 1998, p. 249, figs. 107, 3–4, 108, 1–3, Johnson 2011, p. 593, plate 148 B).

Vegetative cells 42–52 μm wide, 80–270 μm long; transverse walls plane; chloroplasts 3 to 4 per cell (Fig. 6C); multicellular branched rhizoidal outgrowths present. Conjugation scalariform, tubes formed by both gametangia, sometimes predominantly by the male gametangia; fertile gametangia shortened and inflated on both sides, or more strongly on the conjugation side (Fig. 6D). Zygospores ovoid, 52–63 × 62–104 μm; mesospore brown, thick, multilayered, reticulate or finely wrinkled (Fig. 6G). The mesospore sculpture was complex and highly variable during the development of the zygospores.

Notes: SEM observations showed a three-layered mesospore with an outer layer thin and hyaline, and two inner, pigmented layers, of which the inner sublayer was finely reticulate (Simons et al. 1982). The inside of this inner sculptured mesospore layer had spongy structure under SEM (Simons et al. 1982). According to Rundina (1998) the mesospore of this species was poorly studied, and probably
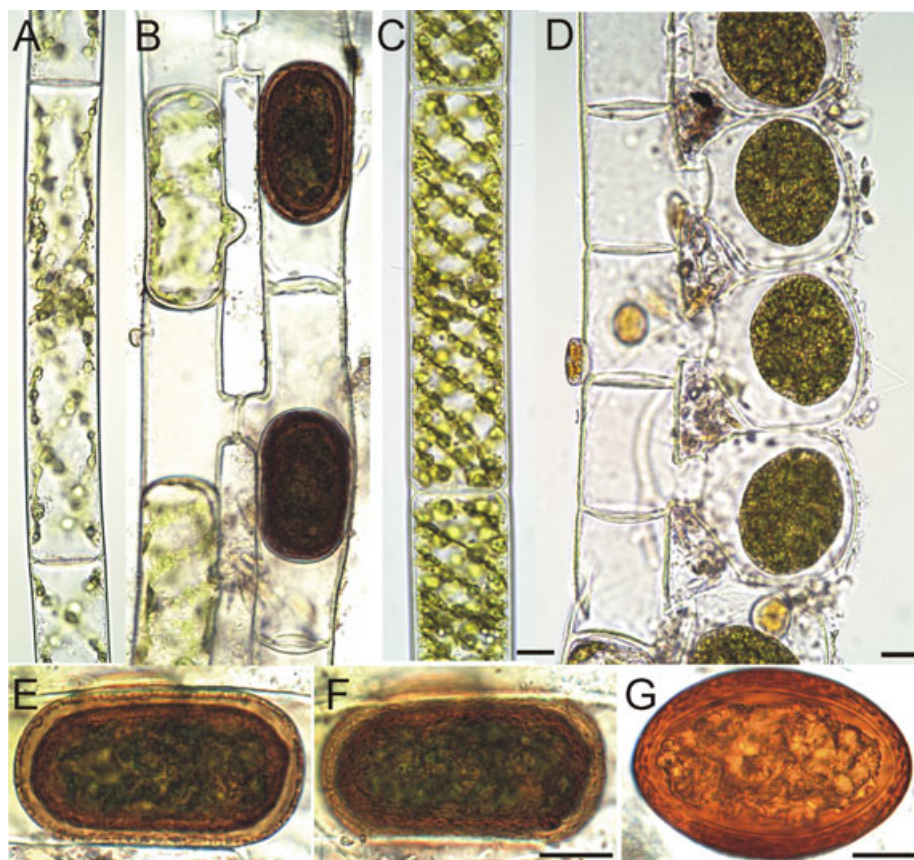
Fɪɢ. 6. Light microscopic images of *Spirogyra* species from CA included in Clade III of phylogenetic analysis: (A and B) *S. notabilis*; (C and D) *S. fluviatilis*; (E) *S. notabilis* zygospore with colorless exospores and two-layered mesospore; (F) same zygospore as above focused on surface of inner reticulate and finely verrucose mesospore layer; (G) *S. fluviatilis* zygospore with multilayered sculptured mesospore. Scale bar, 20 μm.

consisted of four layers. There were discrepancies about the filament width of *S. fluviatilis*, which was reported in the range of 30–45 μm by most authors (Transeau 1951, Kadłubowska 1984, Johnson 2011), but Czurda (1932) considered the width of 26–29 μm or 36–40 μm, Gauthier-Lièvre (1965) 26–35 μm, and Rundina (1998) extended the width range from 26 to 50 μm.

Distribution: Widely distributed in Europe, America, Asia, Africa, Australia (Transeau 1951, Johnson 2011). In the studied area, recorded in twelve localities across CA (watershed regions 1, 3, 5 and 9).

Phylogenetic and taxonomic consideration: Molecular analysis showed that *S. fluviatilis* from CA was genetically identical with *S. africana* from Korea identified by Kim et al. (2004). *S. africana* had been described as variety of *S. fluviatilis* by Fritsch (1921), and elevated to species by Czurda (1932) based on larger filament width (50–60 μm), shape or fertile gametangia, and shape and sculpture of zygospores. However, the mesospore sculpture of *S. africana* and *S. fluviatilis* studied by SEM was very similar (Simons et al. 1982, Kim et al. 2004). In addition, both species showed significant overlap in the filaments

width and zygospore size of strains from CA and Korea included in this study.

*Clade V: **Spirogyra teodoresci** Transeau* (Fig. 7, A and B; Transeau 1951, p. 153, plates XXI, fig. 8, Kadłubowska 1984, p. 286, fig. 439, Rundina 1998, p. 203, fig. 86, 8–11).

Synonym: *Spirogyra varians* f. *minor* Teodor.

Vegetative cells 29–35 μm wide, 40–108 μm long; transverse walls plane; chloroplast one or two per cell (Fig. 7A). Vegetative filaments occasionally have unicellular or multicellular rhizoidal outgrowths and short branches. Conjugation scalariform, rarely lateral, tubes formed by both gametangia; fertile gametangia strongly inflated on the conjugating side (Fig. 7B); some of the vegetative cells swollen. Conjugation sometimes happened between three cells. Zygospores ellipsoid, 26–33 × 35–50 μm; mesospore yellow-brown, smooth. In some specimens the inner mesospore layer appeared finely granulated or striated.

Notes: SEM observations showed a two-layered mesospore with an outer layer thin and hyaline and inner layer pigmented and finely reticulate (Simons et al. 1982). According to Transeau (1951)
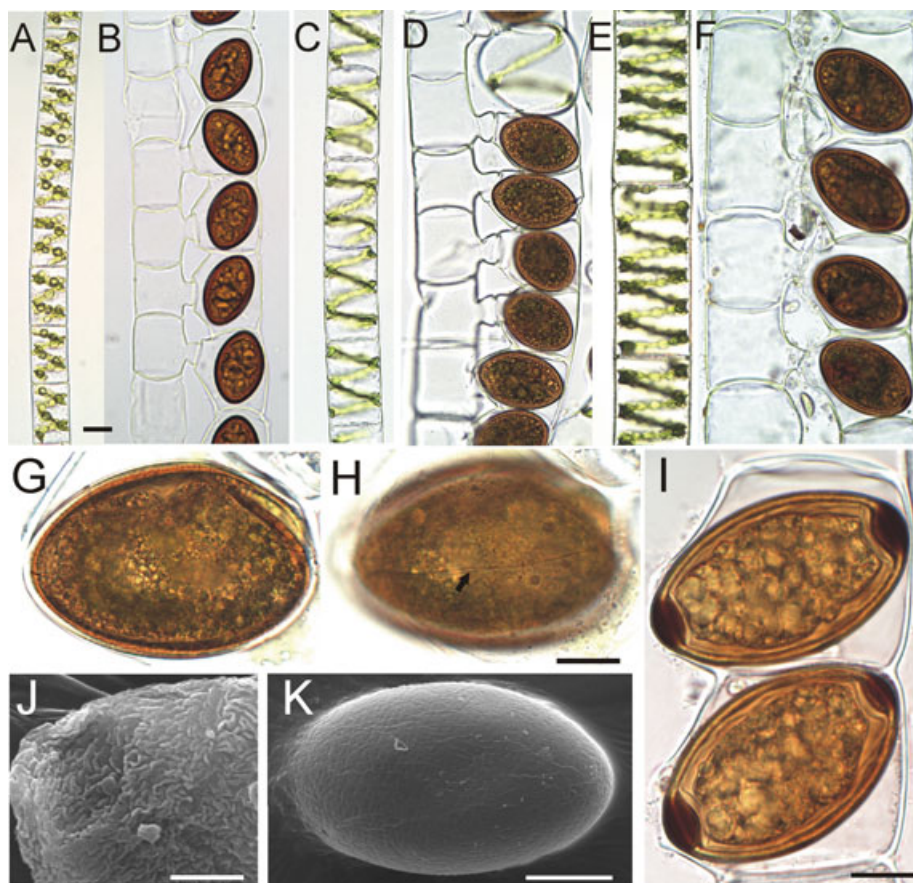
FIG. 7. Light microscopic and scanning electron microscope (SEM) images of *Spirogyra* species from CA included in Clade V of phylogenetic analysis: (A and B) *S. teodoresci*; (C and D) *S. varians*; (E–K) *S. juliana*; (E) filament with plane transverse walls; (F) conjugation; (G) zygospore with colorless exospores, and yellow-brown, thick, finely corrugated mesospore; (H) same zygospore as above focused on finely corrugated mesospore surface and suture (arrow); (I) zygospores KOH treated showed brown cap-like thickenings on poles; (J) SEM image of sculpture on inner mesospore layer; (K) SEM images of zygospore with smooth thin outer mesospore layer wrapped around thick inner sculptured mesospore layer. Scale bar, (J) 5 μm; (A–K) 20 μm.

S. teodoresci is probably included in many records of *S. varians* (Hassall) Kütz. from which it is distinguished by its smaller dimensions throughout.

Distribution: This species is reported from the United States, Europe and Asia (Transeau 1951, Jao 1988, Kargupta and Jha 2004). *S. teodoresci* was identified from nine localities across CA (watershed regions 1, 2, 6, 8 and 9).

Spirogyra varians (Hassall) Kütz. (Fig. 7, C and D) (Transeau 1951, p. 153, plates XXII, fig. 1, Gauthier-Lièvre 1965, p. 163, plate LXI, a, Kadłubowska 1984, p. 288, fig. 443, Rundina 1998, p. 200, figs. 82, 85, 86, Johnson 2011, p. 601, plate 147 G).

Vegetative cells 35–40 μm wide, 40–203 μm long; transverse walls plane; chloroplast one or two per cell (Fig. 7C). Vegetative filaments occasionally have unicellular or multicellular rhizoidal outgrowths. Conjugation scalariform, rarely lateral, tubes formed by both gametangia; fertile gametangia inflated predominantly on the conjugating side (Fig. 7D); some of the sterile cells swollen. Zygospores ellipsoid, rarely ovoid, 35–40 × 50–62 μm; mesospore yellow-brown, smooth. In some speci-

mens the inner mesospore layer appeared finely granulated.

Notes: SEM observations showed a two-layered mesospore with an outer layer thin and hyaline, and inner layer pigmented and with little bulbs (Simons et al. 1982).

Distribution: Widely distributed species in Europe, America, Asia, Africa, Australia (Transeau 1951, Jao 1988). In the studied area recorded in thirteen localities across CA (watershed regions 1, 2, 4, 5 and 9).

Spirogyra juliana Stancheva, J. D. Hall, McCourt et Sheath **sp. nov.** (Fig. 7, E–K).

Diagnosis: Vegetative cells 52–64 μm wide, 56–120 μm long, transverse walls plane. Chloroplast one per cell, making 2.5–6 turns (Fig. 7E). Conjugation scalariform, short tubes formed by both gametangia. Fertile receptive gametangia enlarged, or shortened and inflated on the conjugative side up to 85 μm (Fig. 7F). Vegetative cells between the gametangia sometimes swollen. Zygospores ellipsoid, 41–63 μm wide, 65–93 μm long, completely filling the gametangia, and placed along

the longitudinal, or transverse axis or oblique. Exospore smooth and colorless. Mesospore yellow-brown, two-layered, outer layer (Fig. 7, G and K) thin and hyaline tightly wrapped around the thick, pigmented finely corrugated or reticulate inner mesospore layer (Fig. 7, H and J); suture distinct located in the inner mesospore layer (Fig. 7H). Brown cap-like thickenings of the mesospore were observed on the poles, or laterally in some zygospores (Fig. 7I).

Holotype: Specimen UC1999502 deposited in University Herbarium at University of California, Berkeley (UC).

Type locality: Pine Valley Creek (32°85′ N, 116°52′ W) crossed by Pine Creek Road about 2 km north of Old Hwy 80; Tijuana River watershed, San Diego County, CA, USA. June 06, 2008.

Type strain: RSS015, from culture.

Etymology: The epithet refers to the city of Julian, CA, near Santa Ysabel Creek (33°12′ N, 116°67′ W), where *S. juliana* was first observed in 2008 together with *Zygnema aplanosporum* Stancheva, J. D. Hall et Sheath (Stancheva et al. 2012).

Related species. In respect to mesospore sculpture, *S. juliana* is close to the group of taxa with narrower filaments which do not exceed 44 μm, such as *S. daedalea* Lagerh., *S. daedaleoides* Czurda, *S. westii* Transeau, *S. nodifera* O. et W. Bock, *S. pseudonodifera* O. et W. Bock. Overall, *S. rugulosa* Iwanoff and *S. labyrinthica* Transeau resembled most closely *S. juliana* in respect to filament width and zygospore shape and size. However, in *S. rugulosa* conjugation tubes are formed by male gametangia, and the mesospore structure is questionable, described as finely punctate by Transeau (1951) and corrugated with small pits by Rundina (1998). In *S. labyrinthica* the mesospore is distinctly reticulate. This species is poorly known, without illustration and considered as a synonym of *S. rugulosa* by some authors (Rundina 1998). For details see Table S3.

Notes: In all studied populations of *S. juliana* (see below) zygospores with brown thickening were observed (Fig. 7I). Reith (1980) discussed similar findings of brown thickenings of the mesospore (mostly, but not always located at the poles) in *S. nodifera*, *S. pseudonodifera*, *S. bicaliptrata* Czurda and expressed some doubts concerning a higher taxonomic significance of these structures, which may be originated from environmental influences. Rundina (1998) shared the same opinion about the low-taxonomic value of these ambiguous zygospore structures.

Our observations showed that a chytrid fungus parasitized the zygospores but not the vegetative cells in all populations of *S. juliana*. We did not assess the generic affinities of the chytrid parasite because of the difficulties in observing the zoosporangial operculum, a structure of significance in separating *Chytridum* from *Blyttiomyces* (Chytridiomycota; Blackwell et al. 2011). The parasite fungus aborted the affected host zygospores, while the remaining zygospores in the same filament were either with or without brown thickening.

Distribution and ecology. This species was recorded from a few stream sites in Cleveland National Forest at elevations 896–1128 m a.s.l. Four stream locations were in Tijuana River watershed: Indian Creek (32°89′ N, 116°50′ W) and Noble Canyon Creek (32°86′ N, 116°51′ W), both running into Pine Valley Creek, and Kitchen Creek (32°75′ N, 116°45′ W), and one in San Dieguito River watershed – Santa Ysabel Creek. The reproducing filaments were collected between May 04 and June 10 of 2008, 2009 and 2010. Water chemistry parameters in the type locality Pine Valley Creek and Santa Ysabel Creek, measured in the summer of 2008 were pH 7.4–8.3; temperature 18°C–21.8°C; conductivity 403–452 μS · cm^{-1}; total dissolved phosphorus 0.015–0.026 mg · L^{-1}; total dissolved nitrogen 0.059–0.141 mg · L^{-1}, sulfate 61–71 mg · L^{-1}. *Spirogyra juliana* was associated with *Nostoc verrucosum* Vaucher ex Bornet et Flahault, *Paralemanea catenata* (Kütz.) M. L. Vis et Sheath, *Batrachospermum boryanum* Sirodot, as well as with other zygnematalean algae.

Clade VII: **Spirogyra majuscula** Kütz. (Fig. 5, A, B, E, F; Transeau 1951, p. 190, plate XXXI, figs. 14–15, Gauthier-Lièvre 1965, p. 132, plate XXXXII, c–c″, Kadłubowska 1984, p. 326, fig. 711, Rundina 1998, p. 264, fig. 114, 8–13, Johnson 2011, p. 597, plate 148 E).

Synonym: *Degagnya majuscula* (Kütz.) A. Conard.

Vegetative cells 60–75 μm wide, 70–450 μm long; transverse walls plane; chloroplasts 5 to 8 per cell (Fig. 7A); multicellular rhizoidal outgrowths present. In one population filaments were covered by a pectic layer up to 3 μm (Fig. 5A). Conjugation scalariform, tubes formed by both gametangia; fertile gametangia shortened and inflated on the outer side (Fig. 5B). Zygospores lenticular, 52–70 × 52–70 × 46–53 μm; mesospore brown, smooth with finely striated inner layer (Fig. 5E). Suture prominent, visible on the zygospore front-view (Fig. 5F).

Notes: SEM observations showed that the mesospore is three-layered with outer layer thin and hyaline, and two inner pigmented layers (Simons et al. 1982). The inner sublayer, which appeared finely striated in light microscope showed loose spongy structure under SEM and the suture is placed in this layer (Simons et al. 1982).

Distribution: Widely distributed species in Europe, America, Asia, Africa, and Australia (Transeau 1951, Johnson 2011). In the studied area recorded in three localities in southern CA (watershed regions 4 and 9).

Spirogyra submaxima Transeau (Fig. 5, C, D, G, H; Transeau 1951, p. 191, Gauthier-Lièvre 1965, p. 158, plate LVII, a, Kadłubowska 1984, p. 325, fig. 708, Rundina 1998, p. 265, fig. 115, 2).

Synonym: *Degagnya submaxima* (Transeau) A. Conard.

Vegetative cells 95–105 μm wide, 100–400 μm long; transverse walls plane; chloroplasts 6–8 per cell; filaments covered by pectic layer up to 10 μm in thickness (Fig. 5C); multicellular rhizoidal outgrowths present. Conjugation scalariform, tubes formed by both gametangia; fertile gametangia cylindric (Fig. 5D). Zygospores lenticular, 83–95 × 83–95 × 55–69 μm; mesospore brown, smooth, with finely striated or pitted inner layer (Fig. 5G). Suture prominent, visible on the zygospore front-view (Fig. 5H).

Notes: SEM observations showed that the mesospore is at least two-layered with inner pitted or granulate layer (Hull et al. 1985).

Distribution: Distributed in Europe, America, Asia, Africa, Australia (Transeau 1951, Jao 1988, Johnson 2011). In the studied area recorded in a single lake in southern CA.

Morphological key to the species of Spirogyra *and* Sirogonium *from California.*

1A.   End walls of cells replicate
2A.   One (or two) chloroplasts per cell
3A.   Mesospore smooth
4A.   Conjugation predominantly lateral; diameter of zygospores (30) 31–43 μm .... *S. grevilleana*
4B.   Conjugation scalariform only; diameter of zygospore (21) 28–35 μm...*S. weberi*
3B.   Mesospore punctate...............................*S. australica*
2B.   Three chloroplasts per cell.......................*S. gratiana**
2C.   Four chloroplasts per cell.......................*S. californica*
1B.   End walls of cells plane
5A.   Chloroplasts typically more than five per cell
6A.   Zygospores ellipsoid (not laterally compressed), chloroplasts 3–6 per cell....................*Sirogonium sticticum**
6B.   Zygospores lenticular (laterally compressed), chloroplasts 5 to 8 per cell
7A.   Diameter of vegetative cells (50) 60–75 (80) μm; gametangia inflated on the outer side................*S. majuscula*
7B.   Diameter of vegetative cells (70) 95–105 (110) μm; gametangia cylindrical................................*S. submaxima*
7C.   Diameter of vegetative cells (118) 120–150 (μm; gametangia short cylindrical............................*S. maxima**
5B.   Chloroplasts fewer than five per cell
8A.   Mesospore ornamented
9A.   Chloroplasts one per cell
10A.   Diameter of vegetative cells 52–64 μm.............*S. juliana*
10B.   Diameter of vegetative cells 24–30 μm.........*S. punctata**
9B.   Chloroplasts two to four per cell
11A.   Diameter of zygospores (30) 37–44 (57) μm, mesospore two-layered distinctly ornamented.....................*S. notabilis*
11B.   Diameter of zygospores (47) 52–63 (85) μm, mesopore multilayered, ornamentation complex and variable...*S. fluviatilis*
8B.   Mesospore smooth (sometimes finely granulate)
12A.   Gametangia cylindrical or only slightly inflated
13A.   Three chloroplasts per cell.......................*S. triplicata**
13B.   One or two chloroplasts per cell
14A.   Diameter of vegetative filaments less than 40 μm
15A.   Zygospore ellipsoid to ovoid.......................*S. longata**
15B.   Zygospores polymorphic - globose, ovoid, ellipsoid, cylindrical...*S. lutetiana*
15C.   Zygospores and aplanospores ellipsoid, smaller ....*S. juergensii**
14B.   Diameter of vegetative cells more than 40 μm ....*S. porticalis**
12B.   Gametangia notably inflated
16A.   Gametangia inflated opposite conjugation tube ....*S. borgeana*
16B.   Gametangia inflated toward conjugation tube

17A.   Gametangia slightly inflated or enlarged............ ....*S. catenaeformis**
17B.   Gametangia strongly inflated
18A.   Diameter of zygospores (22) 26–33 (38) μm ....*S. teodoresci*
18B.   Diameter of zygospores (25) 35–40 μm ....*S. varians**

An asterisk indicates that the species was previously reported from CA. Measurements are those observed in Californian material. Reported size ranges are indicated using parentheses.

DISCUSSION

Spirogyra of California. Understanding regional diversity is fundamental to studies of environmental health. Such studies will become increasingly important as water resources become ever more impacted by human activity and climate change. This study added twelve *Spirogyra* species to the algal flora of CA and documented three of the previously recorded taxa. Discovery of such unreported diversity is common with freshwater algae. Our own studies of *Zygnema* C. Agardh from this region added eight new species to the Californian flora, including two new to science (Stancheva et al. 2012). Similar studies of lotic ecosystems in New Zealand doubled the number of species belonging to *Spirogyra* and *Zygnema* known to occur in that region (Novis 2004). Moreover, Zygnematophyceae are better studied in lentic habitats (ditches, ponds, and lakes) (Gerrath 2003) while our study focused on running, neutral to alkaline waters, with relatively high-water velocity. Sterile filaments of *Spirogyra* were recorded in over 15% of stream sites studied across CA, but sexually reproductive populations that allowed species identification were much less frequent and observed mostly in southern CA. This pattern was probably due to the warmer water and shorter growing season known to favor sexual reproduction in *Spirogyra* (Rundina 1998).

The Californian streams we studied were under variable degrees of human influence, and *Spirogyra* species were distributed in localities with contrasting ecological conditions. For instance, *S. borgeana, S. grevilleana, S. juliana, S. teodoresci, S. varians,* and *S. weberi,* occurred in mountain streams with low nutrient concentrations dominated by other algae regarded as indicative of good water quality, such as the red algae *P. catenata* and *B. boryanum,* and the nitrogen-fixing cyanobacteria *N. verrucosum* and *Calothrix* spp. (Komárek et al. 2002, Sheath 2003). Accordingly, Reith (1988) and Johnson (2011) considered many of the above species, such as *S. australica, S. grevilleana, S. teodoresci, S. varians,* and *S. weberi,* characteristic for streams in European mountain areas. In contrast, *S. californica* was restricted to a single stream watershed with elevated ion content and extremely high sulfate concentra-

tions (see Results). *Spirogyra longata* was associated with streams in urban areas, in accordance with the data about its tolerance to elevated water salinity and organic compounds (Rundina 1998, Johnson 2011) and was encountered together with *S. californica.*

Similarly, ecological studies of *Spirogyra* from lakes, ponds, ditches, and slowly flowing rivers in the Netherlands (Simons and Van Beem 1990) and Austria (Hainz et al. 2009) showed that particular species were adapted to nutrient-poor conditions, whereas other species were pollution tolerant. According to Hainz et al. (2009) filament morphotype occurrence was correlated with available water nutrients: taxa with greater filament widths preferred elevated nutrient conditions. More studies are needed to improve our knowledge about nutritional ecophysiology, environmental preferences and indicator value of zygnematophyceans compared to the other green macroalgae (Simons 1994), and particularly with respect to their application in stream biomonitoring.

Taxonomic implications. Taxonomy and classification of *Spirogyra* and filamentous Zygnemataceae has been greatly influenced by a series of large monographs (Czurda 1932, Transeau 1951, Kadłubowska 1984, Rundina 1998). However, our understanding of the diversity and distribution of *Spirogyra* species remains very incomplete. New species continue to be discovered whenever regions are thoroughly studied (e.g., this study, Ferrer and Cáceres 1995, 2008, Kargupta and Jha 2004, Stancheva et al. 2012). Taxonomy of filamentous Zygnemataceae is further complicated by the small number of observable characteristics, the variation within those characteristics (particularly characteristics of the vegetative cells), the necessary reliance on rare reproductive material for identification and the existence of polyploid species groups (McCourt et al. 1986, 2000, Wang et al. 1986, Hoshaw et al. 1987, McCourt and Hoshaw 1990, Kargupta and Jha 2004).

Because of these difficulties, it is not surprising that the existing infrageneric classification of *Spirogyra* classification (Kadłubowska 1984) is not consistent with our molecular phylogeny and does not correspond to natural groups. For instance, all species with replicate transverse walls, separated in section *Salmacis* by Kadłubowska (1984), were grouped together with *Spirogyra* species with plane walls in clade I. Members of section *Conjugata*, group *Maxima* were split out into two clades in the molecular phylogeny, one of which contained *Sirogonium* species as well. This finding makes it difficult to predict to which clade a species belongs without the use of molecular data.

Using a combination of structural and molecular data in systematic and biodiversity studies may allow for more accurate and consistent species identification. However, using molecular data does not eliminate all ambiguity. For example, previous work had indicated that *rbcL* (one of the markers used in this study) may not be sufficiently variable to distinguish between closely related species of some green algae (Hall et al. 2010). In addition, most species-level relationships in *Spirogyra* have not been tested using molecular phylogenetic methods.

Clade III shows how complicated morphological delineation of structurally similar species can be without molecular data, especially when the original species descriptions are not adequate. Clade III included 15 strains with filaments ranging in width from 30 to 57 μm, with three to five chloroplasts per cell, and with sculptured mesospores. Seven different species names were assigned to these strains, but only three unique *rbcL* sequences were detected. Five species (i.e., *S. chungkingensis*, *S. mienningensis*, *S. notabilis*, *S. occidentalis*, and *S. quadrilaminata*) from Arizona shared identical *rbcL* sequences (Drummond et al. 2005) with *S. notabilis* from CA (RSS008, this study). After examining original material of *S. notabilis* we extended the size range of its zygospores (see Results). Based on examination of published and unpublished micrographs of these strains, we think that these strains might represent one highly variable species and that the various determinations may have been made on spores at different developmental stages. Additional study of zygospores of these strains would be necessary to confirm their identification. Should their identities be confirmed, type material from these species – or specimens collected near the type localities – would be needed to determine if these are unique species or all synonyms of *S. occidentalis*, which has priority.

A second group of strains with identical *rbcL* sequences in clade III was *S. fluviatilis* from CA and *S. africana* (KCH 1–4) from Korea (Kim et al. 2004, 2006). These two species were structurally very similar to one another. Based on the *rbcL* sequences, they may be closely related but distinct species, or they could possibly represent a species complex or polyploid series having identical, or nearly identical, *rbcL* sequences with filaments of different average widths. Although the molecular and morphological evidence are consistent with one another, the relationship between *S. africana* and *S. fluviatilis* remains unclear. This trend is due partly to the variability within *S. fluviatilis.*

Spirogyra fluviatilis is a widespread and structurally variable species often found in running water (Transeau 1951). For instance, "*S. fluviatilis*"–like filaments with considerable width variation (24–50 μm) were found to grow in the Daly River, Australia, and were considered as three morphotypes within the *S. fluviatilis* complex in a study of nutrient limitation of this species (Townsend et al. 2008). Our results from San Diego River and Matilija River in CA showed that only the larger filaments (>45 μm width) shared identical *rbcL* sequences with *S. fluviatilis* (RSS030 and RSS033), while the narrower

filament (<40 μm) from the same collections belonged to another taxon (RSS029 and RSS034). This apparently variable species may represent many different species even within a single habitat.

Our molecular systematic data do help clarify some taxonomic questions. For example, we determined that two structurally very similar species, *S. varians* and *S. teodoresci*, which differed morphologically only by the smaller dimensions of the latter species (Transeau 1951) were genetically distinct (Fig. 1). This finding was unexpected since some species concepts lowered *S. teodoresci* to a variety of *S. varians* (e.g., Rundina 1998). Similarly, *S. longata* and *S. lutetiana*, which were considered synonymous with *S. decimina* by Rundina (1998), were determined to be distinct species and very distant from *S. decimina* from Korea (Kim et al. 2004). This may be due to misidentification of *S. decimina*-like species (although it could also be due to biogeographic variation, see discussion below). For example, many discrepancies exist between different studies about the number of chloroplasts in *S. decimina*. According to Rundina (1998), there are one or two chloroplasts in *S. decimina*, whereas Kim et al. (2004) described *S. decimina* from South Korea with two to four chloroplasts, which could explain the distant phylogenetic position of this strain from Californian strains of *S. longata* and *S. lutetiana*, both with one or two chloroplasts. Although considerably more molecular sampling would be necessary before any definite conclusion about the relationship between molecular and morphological species identification in *Spirogyra* can be made, our results suggest that one should interpret reported distributions of morphologically identified species with caution.

Many species of *Spirogyra* are reportedly globally distributed. By sampling outside CA (the main study area) and incorporating sequences from GenBank, we were able to gain a better understanding of the larger biogeographic patterns of *Spirogyra* species. Previous phylogenetic studies included very few representatives of widely distributed species. Drummond et al. (2005) studied several strains of a few *Spirogyra* species (e.g., *S. communis* and *S. pratensis*) and reported that most strains identified as each species had identical *rbcL* sequences. However, these strains were derived from small geographic areas and represented polyploidy species groups (Hoshaw et al. 1985, Drummond et al. 2005). By contrast, *S. pratensis* UTEX 1746 from India was only distantly related to *S. pratensis* from the USA (Drummond et al. 2005). Studies by Kim et al. (2006) also included many strains of some species from Korea. They reported that the Korean strains of each species had similar *rbcL* sequences and that these strains were closely related – although not always identical – to strains of those species from other parts of the world (Kim et al. 2006).

In this study, we included a much broader sampling of strains than had been studied previously and we observed a slightly different pattern of *Spirogyra* global diversity. For example, our strains of *S. grevilleana* (RSS011 and RSS017) were closely related to each other but quite different from *S. grevilleana* UTEX 477 from England (Fig. 1). Similarly, our strain of *S. weberi* RSS004 (from CA, USA) was only distantly related to *S. weberi* KCH 218 (from South Korea), and our strains of *S. maxima* (RSS026, RSS028, and RSS032; from CA, USA) are distant relatives of *S. maxima* UTEX 2495 from Florida, USA. Although there were undoubtedly irregularities in terms of identification, this pattern suggests that species with similar structural characteristics from different parts of the world are sometimes only distantly related to one another. Even when closely related, strains identified as the same species from different parts of the world often had different *rbcL* sequences. For example, three strains of *S. varians* – one from England (UTEX479) and two from South Korea (KCH 96-4 and KCH 96-4) – had similar but not identical *rbcL* sequences, and were closely related to *S. varians* from CA (RSS013). Also, *S. tenuissima* from the USA (JH1015) and Portugal (ACOI 1925) did not share identical sequence. Based on currently available data, it is unclear how this apparent intraspecific variation correlates to biological species or biogeographic patterns. Because many cultured strains will no longer reproduce sexually, their identity cannot be confirmed and their reproductive characteristics cannot be reevaluated in the same manner as our more recent collections. Also, we do not have sufficient data on any species to understand the amount of genetic diversity that is to be expected within a population or any larger geographic area. Nonetheless, one must conclude that either there is considerable genetic variation within some widely distributed species or structurally similar strains from different continents are likely to be phylogenetically distinct species.

Collectively, our data suggest that *Spirogyra* may possess a great deal of cryptic diversity. That is, morphologically similar strains with widely divergent gene sequences, or, conversely, diverse morphotypes that share identical or nearly identical sequences for one or more genes. Given the degree of phenotypic variability known in the genus, clades based on derived sequence characters constitute a preferred basis for delineating species or species groups (i.e., closely related taxa that may be members of a population gene pool). Reconciliation of identifications based on morphology of vegetative and reproductive characters with monophyletic groups based upon genetic data will be a major challenge for systematics of *Spirogyra*, as well as other taxa in the Zygnematophyceae.

Our molecular phylogenetic results showed that *Sirogonium* species are intermixed with *Spirogyra* species (Figs. 1 and 2) in clade II, which indicates that '*Sirogonium*' is not distinct from *Spirogyra* as currently circumscribed and could possibly be considered a

subgenus of *Spirogyra.* However, a second clade with *Sirogonium*-like chloroplasts has been identified. This lineage (clade VII) was both phylogenetically distinct and structurally homogenous. *Spirogyra majuscula* and *S. submaxima* from this clade were placed as members of the genus *Degagnya* A. Conard (Conard 1936a,b,c,d, Conard and Conard 1943). We continue to study these species to better understand their structural characteristics.

Considering that *Spirogyra* (as it is currently circumscribed) originated soon after the divergence of conjugating green algae from other charophytes (McCourt et al. 2000, Karol et al. 2001, Gontcharov et al. 2003, 2004, Hall et al. 2008) and the phyletic and structural diversity within *Spirogyra,* it would be reasonable to divide *Spirogyra* into several genera. However, given the diversity of the genus and limited sampling to date, the phylogenetic, structural and geographic limits of some clades of *Spirogyra* remain obscure. Although there are some patterns among the clades with regards to filament diameter, number of chloroplasts, the number of turns of the chloroplast per cell, and mesospore sculpture, there is considerable overlap among the clades. Further, each clade contained species with one or two chloroplasts and plane transverse walls, except for clade VII. It appears that many of the vegetative characteristics of *Spirogyra* have evolved multiple times in parallel.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Maximum Likelihood tree based on a combined analysis of ATP synthase beta subunit 3 (*atpB*) and RUBISCO large subunit (*rbcL*) found using RAxML with the data set partitioned by gene. Numbers above branches are bootstrap values from Parsimony, RAxML and posterior probabilities from Bayesian analysis, respectively. An asterisk (*) represents a bootstrap value of 100 or a posterior probability of 1.0. A dash (-) represents a bootstrap value of less than 50 or a posterior probability of less than 0.50. Californian strains are indicated in gray.

Table S1. Strains used in phylogenetic study. Genbank numbers KC779063-KC779224 were newly determined in this study.

Table S2. Structural characteristics of investigated strains. Most measurements and observations were based on material from our collections and do not reflect the known diversity of the named species. Structural information in brackets was gleaned from the literature – primarily Transeau 1951 and Kadłubowska 1984.

Table S3. Comparison of vegetative and reproductive features of *Spirogyra californica* and *S. juliana* with related taxa. Information for other species was summarized from the literature – primarily Transeau 1951 and Kadłubowska 1984.